

Complement inhibitors

The present invention relates to complement inhibitors that inhibit both the classical and alternative complement pathways. In particular, the invention relates to complement inhibitors derived from the salivary glands of haematophagous arthropods that inhibit both the classical and alternative complement pathways. The invention also relates to the use of such complement inhibitors in the treatment and prevention of diseases.

All documents mentioned in the text and listed at the end of this description are incorporated herein by reference.

Complement proteins form the principal arm of the effector immune system (Law and Reid, 1995; Dodds and Sim, 1997; Whaley, 1993). More than 30 proteins in serum and at the cell surface are involved in complement system function and regulation. The system is activated by the presence of foreign antigens. Two activation pathways exist: (1) the classical pathway which is activated by IgM and IgG complexes or by recognition of carbohydrates; and (2) the alternative pathway which is activated by non-self surfaces (lacking specific regulatory molecules) and by bacterial endotoxins. The two pathways comprise parallel cascades of events that result in the production of complement activation through the formation of similar C3 and C5 convertases on cell surfaces resulting in the release of acute mediators of inflammation (C3a and C5a) and formation of the membrane attack complex, as shown in Figure 1.

The effects of complement activation are wide ranging and include: initiation of inflammation especially through release of the acute mediators C3a and C5a; opsonisation and phagocytosis of pathogens via deposition of C4b and C3b; clearance of immune cell complexes by recruitment of macrophages; increased efficiency of antigen presentation to B cell receptors through covalent association of antigen and C3d; retention of antigen in germinal centres; enhanced antigen uptake by antigen presenting cells; and membrane attack complex (MAC) mediated disruption of foreign or disordered cells (e.g. bacteria, parasites, tumour cells).

Activation of complement must be tightly controlled to prevent damage to the body's own tissues. Control is mediated by the short half-lives of activated proteins, and by control proteins present in plasma and on cell membranes. When complement control goes awry, damage to body tissue may cause disease. Sahu and Lambris (2000) have compiled a list of 29 pathological conditions in which failure to control complement activation has a role.

They include: acute pancreatitis, Alzheimer's disease, allergic encephalomyelitis, allotransplantation, asthma, adult respiratory distress syndrome, burn injuries, Crohn's disease, glomerulonephritis, haemolytic anaemia, haemodialysis, hereditary angioedema, ischaemia reperfusion injuries, multiple system organ failure, multiple sclerosis, myasthenia gravis, myocardial infarction, psoriasis, rheumatoid arthritis, septic shock, systemic lupus erythematosus, stroke, vascular leak syndrome and xenotransplantation. Data derived from animal models (knockout and transgenic mice) demonstrating the essential role of complement activation in some of these diseases has been reviewed by Ward *et al.*, 2000.

- 10 Tissue damage arising from complement activation is mediated by the MAC and by the anaphylatoxins, C3a and C5a. These two peptides induce damage through their effects on neutrophils, eosinophils, macrophages, microglial cells, basophils and mast cells. Anaphylatoxin stimulated cells release proinflammatory mediators, tissue degradative enzymes, oxygen free radicals and increase adhesion molecule and inflammatory cytokine expression (Ember *et al.*, 1998). This in turn leads to the elaboration of the immune response and activation of haemostatic mechanisms such as coagulation and fibrinolysis. The role of the anaphylatoxins in infectious and non-infectious inflammatory diseases has recently been reviewed by Kohl (2001). The proinflammatory activity of the MAC is chiefly mediated indirectly by induction of cell activation by causing increased expression of adhesion molecules, tissue factor and chemokines.

In view of the importance of the control of complement in the treatment of medical diseases and disorders, numerous complement inhibitors are under development for therapeutic use (Table 1). None of these inhibitors are yet available in the clinic although some are currently in phase I/II clinical trials. The inhibitory molecules under development are high molecular weight natural inhibitors (Hebell *et al.*, 1991; Weisman *et al.*, 1990) that are often specifically engineered (Mulligan *et al.*, 1999; Smith and Smith, 2001; Zhang *et al.*, 2001). They are generally antibodies directed at specific complement components (Frei *et al.*, 1987; Link *et al.*, 1999), small molecules including RNA aptamers (Biesecker *et al.*, 1999) or molecules that specifically target complement receptors.

Table 1 (from Sahu and Lambris, 2000): Complement inhibitors under development

Inhibitor	Target
Protein	
C1-Inh	C1
SCR1	C3b, C4b, C3bBb, C3b ₂ Bb, C4b2a, C4b3b2a
Vaccinia CCP	C3b, C4b, C3bBb, C3b ₂ Bb, C4b2a, C4b3b2a
SDAF	C3bBb, C3b ₂ Bb, C4b2a, C4b3b2a
SMCP	C3b, C4b
SMCP-DAF	C3b, C4b, C3bBb, C3b ₂ Bb, C4b2a, C4b3b2a
SCD59	C5b – 8
DAF-CD59	C3b, C4b, C3bBb, C3b ₂ Bb, C4b2a, C4b3b2a, C5b – 8
C5a mutants	C5aR
Anti-C5 antibody	C5
Anti-C3 antibody	C3
Anti-C5a antibody	C5a
Anti-C3a antibody	C3a
Small molecule	
N MeFKPdChaWdR	C5aR
F-(OpdChaWR)C5aR	
Compstatin	C3
RNA aptamer	C5
BCX-1470	Factor D
FUT-175	C1s, Factor D, C3bBb, C3b ₂ Bb, C4b2a, C4b3b2a
K-76	C5
Thioester inhibitors	C3, C4

In view of the importance of complement inhibitors in the treatment of a wide range of diseases and conditions, there remains a need for additional complement inhibitors.

5 Summary of the invention

According to a first aspect of the invention, there is provided a complement inhibitor molecule that inhibits both the classical and the alternative pathways of complement activation.

- By "inhibit" is meant that the effect of the alternative and classical pathways of complement activation is reduced. The ability of a molecule to reduce the effect of the classical complement pathway and the alternative complement pathway can be determined by standard haemolytic assays known in the art, such as those described in the Examples and in Giclas *et al* (1994). Preferably, the presence of a complement inhibitor molecule of the invention reduces red blood cell lysis in standard haemolytic assays for the classical and alternative pathways of complement activation by at least 20% compared to a standard assay in the absence of a complement inhibitor molecule, more preferably by at least 30%, 40%, 50%, 60%, 70% or 80%.
- 10 Preferably, the complement inhibitor molecule of the invention inhibits cleavage of C5 by the C5 convertase in the classical pathway and the C5 convertase in the alternative pathway. As shown in Figure 1, the conversion of C5 to C5b by C5 convertase occurs in both the alternative complement pathway and the classical complement pathway. The C5 convertase in the classical pathway is C4b3b2a and the C5 convertase in the alternative pathway is C3b₂Bb. The inhibition of C5 cleavage by both these C5 convertases thus inhibits both the classical and the alternative pathways of complement activation. The ability of a molecule to inhibit cleavage of C5 by the C5 convertases of the classical and alternative pathways can be determined by standard *in vitro* assays. Preferably, the presence of a complement inhibitor molecule of the invention reduces cleavage of C5 by the C5 convertases of the classical and alternative pathways by at least 20% compared to a standard assay in the absence of a complement inhibitor molecule, more preferably by at least 30%, 40%, 50%, 60%, 70% or 80%. Preferably, the complement inhibitor molecules of the invention are able to inhibit cleavage of C5 by the C5 convertases of the classical and alternative pathways from a range of mammalian species.
- 25 According to a second aspect of the invention, there is provided a complement inhibitor molecule which inhibits cleavage of C5 by a C5 convertase. The complement inhibitor molecule according to this aspect of the invention may inhibit the cleavage of C5 by the C5 convertase of the classical pathway of complement activation. Alternatively, the complement inhibitor molecule of this aspect of the invention may inhibit cleavage of C5 by the C5 convertase of alternative pathway of complement activation. The ability of a molecule to inhibit cleavage of C5 by the C5 convertases of the classical or alternative pathways can be determined by standard *in vitro* assays as described above. Preferably, the presence of a complement inhibitor molecule of the invention reduces cleavage of C5 by

the C5 convertases of the classical or alternative pathways by at least 20% compared to a standard assay in the absence of a complement inhibitor molecule, more preferably by at least 30%, 40%, 50%, 60%, 70% or 80%.

The complement inhibitor molecules of the invention may inhibit cleavage of C5 by the C5 convertases of the classical pathway, the alternative pathway or both the classical and alternative pathways by direct binding to either C5 or to either or both of the C5 convertases. Preferably, the complement inhibitor molecules of the invention inhibit cleavage of C5 by direct binding to C5. Alternatively, the complement inhibitor molecules may inhibit cleavage of C5 by binding to a complex of C5 and a C5 convertase. The invention further provides a complement inhibitor molecule complexed with C5, complexed with a C5 convertase, or complexed with both C5 and a C5 convertase. The C5 convertase in these complexes may be a C5 convertase of either the classical or alternative pathway.

Preferably, the complement inhibitor molecules is derived from a haematophagous arthropod. The term "haematophagous arthropod" includes all arthropods that take a blood meal from a suitable host, such as insects, ticks, lice, fleas and mites.

Complement is one of the first immune defence systems encountered by blood sucking ticks when they attempt to feed. If feeding ticks do not assume rapid control of complement activation, they may be damaged by the host's inflammatory response. An 18.5 kDa protein from the tick *Ixodes scapularis* that inhibits the alternative pathway of complement activation has been cloned and expressed (Valenzuela *et al.*, 2000). Complement inhibitory activity has also been described in *Dermacentor andersoni* (Ribeiro, 1987) and *Ornithodoros moubata* (Astigarraga *et al.*, 1997) salivary gland extract but the active components have not been identified. Molecules that inhibit both the alternative and classical complement pathways have not previously been identified in ticks.

When the complement inhibitor molecule of the invention is derived from a haematophagous arthropod, it is preferably derived from a tick. Preferably, the complement inhibitor molecule is derived from the tick *Ornithodoros moubata*.

Preferably, the complement inhibitor molecule derived from *Ornithodoros moubata* is a protein comprising amino acids 19 to 168 of the amino acid sequence in Figure 4, or a functional equivalent thereof. In particular, the complement inhibitor molecule is a protein

comprising amino acids 1 to 168 of the amino acid sequence in Figure 4 or a functional equivalent thereof.

The protein having the amino acid sequence given in Figure 4, also referred to herein as “OmCI protein”, was isolated from the salivary glands of the tick *Ornithodoros moubata* and has been found to inhibit the classical and alternative complement pathways. More particularly, it has been found to inhibit cleavage of C5 by the C5 convertases of both the classical and alternative pathways of complement activation, targeting the C5 activation step without affecting C3 activation. The OmCI protein inhibits C5 cleavage by the C5 convertases in a range of mammals. The first 18 amino acids of the OmCI protein sequence given in Figure 4 form a signal sequence which is not required for complement inhibitory activity. The term “OmCI protein”, as used herein, refers to the sequence given in Figure 4 with or without the signal sequence.

The term “functional equivalent” is used herein to describe homologues and fragments of the OmCI protein which retain the ability to inhibit the classical and the alternative complement pathways. Preferably, functional equivalents retain the ability to inhibit cleavage of C5 by C5 convertases of the classical and alternative pathways. Functional equivalents also include homologues and fragments of the OmCI protein which retain the ability to inhibit the classical pathway by inhibiting cleavage of C5 by the C5 convertase of the classical pathway or which retain the ability to inhibit the alternative pathway of complement by inhibiting cleavage of C5 by the C5 convertase of the alternative pathway.

The term “homologue” is meant to include reference to paralogues and orthologues of the OmCI sequence that is explicitly identified in Figure 4, including, for example, the OmCI protein sequence from other tick species, including *Rhipicephalus appendiculatus*, *R. sanguineus*, *R. bursa*, *A. americanum*, *A. cajennense*, *A. hebraeum*, *Boophilus microplus*, *B. annulatus*, *B. decoloratus*, *Dermacentor reticulatus*, *D. andersoni*, *D. marginatus*, *D. variabilis*, *Haemaphysalis inermis*, *Ha. leachii*, *Ha. punctata*, *Hyalomma anatolicum anatolicum*, *Hy. dromedarii*, *Hy. marginatum marginatum*, *Ixodes ricinus*, *I. persulcatus*, *I. scapularis*, *I. hexagonus*, *Argas persicus*, *A. reflexus*, *Ornithodoros erraticus*, *O. moubata moubata*, *O. m. porcinus*, and *O. savignyi*. The term “homologue” is also meant to include the OmCI protein sequence from mosquito species, including those of the *Culex*, *Anopheles* and *Aedes* genera, particularly *Culex quinquefasciatus*, *Aedes aegypti* and

Anopheles gambiae; flea species, such as *Ctenocephalides felis* (the cat flea); horseflies; sandflies; blackflies; tsetse flies; lice; mites; leeches; and flatworms.

Methods for the identification of homologues of the OmCI sequence given in Figure 4 will be clear to those of skill in the art. For example, homologues may be identified by
 5 homology searching of sequence databases, both public and private. Conveniently, publicly available databases may be used, although private or commercially-available databases will be equally useful, particularly if they contain data not represented in the public databases. Primary databases are the sites of primary nucleotide or amino acid sequence data deposit and may be publicly or commercially available. Examples of
 10 publicly-available primary databases include the GenBank database (<http://www.ncbi.nlm.nih.gov/>), the EMBL database (<http://www.ebi.ac.uk/>), the DDBJ database (<http://www.ddbj.nig.ac.jp/>), the SWISS-PROT protein database (<http://expasy.hcuge.ch/>), PIR (<http://pir.georgetown.edu/>), TrEMBL (<http://www.ebi.ac.uk/>), the TIGR databases (see <http://www.tigr.org/tdb/index.html>), the
 15 NRL-3D database (<http://www.nbrfa.georgetown.edu/>), the Protein Data Base (<http://www.rcsb.org/pdb/>), the NRDB database (<ftp://ncbi.nlm.nih.gov/pub/nrdb/README>), the OWL database (<http://www.biochem.ucl.ac.uk/bsm/dbbrowser/OWL/>) and the secondary databases PROSITE (<http://expasy.hcuge.ch/sprot/prosite.html>),
 20 PRINTS (<http://iupab.leeds.ac.uk/bmb5dp/prints.html>), Profiles (http://ulrec3.unil.ch/software/PFSCAN_form.html), Pfam (<http://www.sanger.ac.uk/software/pfam>), Identify (<http://dna.stanford.edu/identify/>) and Blocks (<http://www.blocks.fhcrc.org>) databases. Examples of commercially-available databases or private databases include PathoGenome (Genome Therapeutics Inc.) and
 25 PathoSeq (Incyte Pharmaceuticals Inc.).

Typically, greater than 30% identity between two polypeptides (preferably, over a specified region) is considered to be an indication of functional equivalence and thus an indication that two proteins are homologous. Preferably, proteins that are homologues have a degree of sequence identity with the OmCI protein sequence identified in Figure 4
 30 of greater than 60%. More preferred homologues have degrees of identity of greater than 70%, 80%, 90%, 95%, 98% or 99%, respectively with the OmCI protein sequence given in Figure 4. Percentage identity, as referred to herein, is as determined using BLAST version 2.1.3 using the default parameters specified by the NCBI (the National Center for

Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>) [Blosum 62 matrix; gap open penalty=11 and gap extension penalty=1].

Homologues of the OmCI protein sequence given in Figure 4 include mutants containing amino acid substitutions, insertions or deletions from the wild type sequence, provided that inhibition of the classical or alternative complement pathways demonstrated by the wild type protein sequence is retained. Preferably, the mutants retain the ability to inhibit both the classical and alternative complement pathways. Preferably, such mutants retain the ability to inhibit cleavage of C5 by C5 convertases of both the alternative and classical pathways. Mutants that retains the ability to inhibit either the classical or the alternative complement pathway are also included in the term homologue provided that they retain the ability to inhibit cleavage of C5 by either the C5 convertase of the alternative pathway or by the C5 convertase of the classical pathway. Mutants thus include proteins containing conservative amino acid substitutions that do not affect the function or activity of the protein in an adverse manner. This term is also intended to include natural biological variants (e.g. allelic variants or geographical variations within the species from which the OmCI proteins are derived). Mutants with improved activity in the inhibition of the classical or alternative pathways compared to that of the wild type protein sequence may also be designed through the systematic or directed mutation of specific residues in the protein sequence. Preferably, such mutants show improved inhibition of C5 cleavage by the C5 convertase of the classical pathway or by the C5 convertase of the alternative pathways. Preferably, these mutants show improved inhibition of C5 cleavage by C5 convertases of both the alternative and classical pathways.

Fragments of the OmCI protein and of homologues of the OmCI protein are also provided by the invention. Included as such fragments are not only fragments of the *O. moubata* OmCI protein that is explicitly identified herein in Figure 4, but also fragments of homologues of this protein, as described above. Such fragments of homologues will typically possess greater than 60% identity with fragments of the OmCI protein sequence in Figure 4, although more preferred fragments of homologues will display degrees of identity of greater than 70%, 80%, 90%, 95%, 98% or 99%, respectively with fragments of the OmCI protein sequence in Figure 4. Fragments of the OmCI protein comprising the sequence in Figure 4 and fragments of homologues thereof preferably inhibit the classical and the alternative complement pathways, preferably by inhibiting C5 cleavage by C5 convertases of both the classical and alternative pathways. Fragments of the OmCI protein and of

homologues thereof which inhibit either the classical complement pathway or the alternative complement pathway are also included in the invention, provided that they retain the ability to inhibit the cleavage of C5 by either the C5 convertase of the classical pathway or by the C5 convertase of the alternative pathway. Fragments with improved
5 activity in inhibiting the classical or alternative complement pathways and in particular improved activity in inhibiting C5 cleavage by the C5 convertases may, of course, be rationally designed by the systematic mutation or fragmentation of the wild type sequence followed by appropriate activity assays.

The term "functional equivalent" also refers to molecules that are structurally similar to the
10 OmCI protein or that contain similar or identical tertiary structure, particularly in the environment of the active site or active sites of OmCI. OmCI is thought to inhibit cleavage of C5 by the C5 convertases by direct binding to either C5 or to both of the C5 convertases or to complexes of C5 and the C5 convertases. OmCI is shown in the Examples herein to bind to C5, supporting the suggestion that it inhibits cleavage of C5 by the C5 convertases
15 by direct binding to C5 alone or when C5 is part of a complex with a C5 convertase. Although the applicant does not wish to be bound by this theory, it is postulated that binding of OmCI to C5 may prevent access of the C5 convertases to the C5 cleavage site. Preferred functional equivalents of OmCI therefore include molecules, such as homologues and fragments, that retain the ability to bind directly to C5.

20 OmCI is also believed to be a member of the lipocalin family of proteins that bind small ligands internally. OmCI may therefore also inhibit the cleavage of C5 and or deposition of the MAC indirectly, by binding to a small ligand that would normally bind to C5, C5 convertase or the MAC and that is required for normal function. No small ligands have previously been described as essential for complement system function although the C8
25 gamma component of the MAC is a lipocalin that may bind a small ligand. Functional equivalents thus include molecules that contain similar or identical tertiary structure to the active site(s) in the OmCI protein that binds to C5 or the C5 convertases and/or the active site in the OmCI protein that binds a small ligand. In particular, synthetic molecules that are designed to mimic the tertiary structure or active site(s) of the OmCI protein are
30 considered to be functional equivalents.

The invention further provides the OmCI protein, or a fragment or a functional equivalent thereof, complexed with C5, complexed with a C5 convertase, or complexed with both C5

and a C5 convertase. The C5 convertase in these complexes may be a C5 convertase of either the classical or alternative pathway.

As discussed in more detail previously, there is a continuing need for complement inhibitors and in particular for complement inhibitors that inhibit both the classical and
5 alternative pathways of complement activation. The complement inhibitor molecules of the invention, including the OmCI protein and functional equivalents thereof, will have a wide range of medical applications, in the treatment, prevention and diagnosis of diseases and conditions, as well as being useful research tools in the study of complement inhibition and of the inhibition of both the alternative and classical pathways of complement activation.
10 The OmCI protein itself will be particularly useful for these applications as it inhibits the complement cascade of diverse mammalian species.

The complement inhibitor molecules of the invention, including the OmCI protein and functional equivalents thereof, may be prepared in recombinant form by expression in a host cell. Such expression methods are well known to those of skill in the art and are
15 described in detail by Sambrook *et al* (2000) and Fernandez & Hoeffler (1998). The proteins and fragments of the present invention can also be prepared using conventional techniques of protein chemistry. For example, protein fragments may be prepared by chemical synthesis.

According to a further embodiment, the invention provides an antibody which binds to a
20 complement inhibitor molecule as described above. In particular, the invention provides an antibody which binds to the OmCI protein or a functional equivalent thereof. Antisera and monoclonal antibodies can be made by standard protocols using a complement inhibitor molecule, such as the OmCI protein or functional equivalent thereof, as an immunogen (see, for example, Antibodies: A Laboratory Manual ed. By Harlow and Lane, Cold Spring
25 Harbor Press, 1988). As used herein, the term "antibody" includes fragments of antibodies which also bind specifically to a complement inhibitor molecule. The term "antibody" further includes chimeric and humanised antibody molecules having specificity for a complement inhibitor molecule of the invention. In some cases, it will be desirable to attach a label group to the antibody in order to facilitate detection. Preferably, the label is
30 an enzyme, a radiolabel or a fluorescent tag.

Derivatives of the complement inhibitor molecules described above are also included as embodiments of the invention. In particular, the invention provides derivatives of the

OmCI protein or of functional equivalents thereof. Such derivatives include a fusion protein comprising a complement inhibitor molecule that is genetically or chemically fused to one or more peptides or polypeptides. The purpose of the additional peptide or polypeptide may be to aid detection, expression, separation or purification of the protein or it may lend the protein additional properties as desired. Examples of potential fusion partners include beta-galactosidase, glutathione-S-transferase, luciferase, a polyhistidine tag, a T7 polymerase fragment and a secretion signal peptide. Other potential fusion partners include potential biopharmaceuticals, such as proteins that are being developed for use as drugs to treat specific diseases.

- 10 The complement inhibitor molecule may also be fused to a marker domain. Preferably, the marker domain is a fluorescent tag, an epitope tag that allows purification by affinity binding, an enzyme tag that allows histochemical or fluorescent labelling, or a radiochemical tag. In a preferred embodiment, the marker domain is a radiochemical tag.

Methods for the generation of fusion proteins are standard in the art and will be known to the skilled reader. For example, most general molecular biology, microbiology recombinant DNA technology and immunological techniques can be found in Sambrook *et al.* (2000) or Ausubel *et al.* (1991). Generally, fusion proteins may be most conveniently generated recombinantly from nucleic acid molecules in which two nucleic acid sequences are fused together in frame. These fusion proteins will be encoded by nucleic acid molecules that contain the relevant coding sequence of the fusion protein in question.

According to a further aspect of the invention, there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a complement inhibitor molecule according to the above-described aspects of the invention. Such molecules include single- or double-stranded DNA, cDNA and RNA, as well as synthetic nucleic acid species. Preferably, the nucleic acid sequences comprise DNA.

Preferably, the nucleic acid molecule comprises a nucleotide sequence encoding the OmCI protein or a functional equivalent thereof. Preferably, such a nucleic acid molecule comprises bases 53 to 507 of the nucleotide sequence in Figure 4. This nucleotide sequence encodes the OmCI protein without the signal sequence. The first 54 bases of the nucleotide sequence in Figure 4 encode the signal sequence of OmCI which is not required for complement inhibitory activity. The invention also provides a nucleic acid molecule comprising bases 1 to 507 of the nucleic acid sequence in Figure 4 which encodes the

OmCI protein with the signal sequence. As used herein, the phrase "nucleic acid molecules encoding the OmCI protein" includes both nucleic acid molecules encoding the OmCI protein with the signal sequence nucleic acid molecules encoding the OmCI protein without the signal sequence.

- 5 The invention also includes cloning and expression vectors comprising the nucleic acid molecules of this aspect of the invention. Such expression vectors may incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements, promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the
10 nucleic acid molecules of the invention.

Additionally, it may be convenient to cause a recombinant protein to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion, signalling and processing sequences.

- Vectors according to the invention include plasmids and viruses (including both
15 bacteriophage and eukaryotic viruses), as well as other linear or circular DNA carriers, such as those employing transposable elements or homologous recombination technology. Many such vectors and expression systems are known and documented in the art (Fernandez & Hoeffler, 1998). Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus- based vectors.

- 20 Suitable hosts for recombinant expression include commonly used prokaryotic species, such as *E. coli*, or eukaryotic yeasts that can be made to express high levels of recombinant proteins and that can easily be grown in large quantities. Preferably, the host cell is a eukaryotic yeast cell. Mammalian cell lines grown *in vitro* are also suitable, particularly when using virus-driven expression systems. Another suitable expression system is the
25 baculovirus expression system that involves the use of insect cells as hosts. An expression system may also constitute host cells that have the DNA incorporated into their genome. Proteins, or protein fragments may also be expressed *in vivo*, for example in insect larvae or in mammalian tissues.

- A variety of techniques may be used to introduce the vectors according to the present
30 invention into prokaryotic or eukaryotic cells. Suitable transformation or transfection techniques are well described in the literature (Sambrook *et al*, 1989; Ausubel *et al*, 1991; Spector, Goldman & Leinwald, 1998). In eukaryotic cells, expression systems may either

be transient (e.g. episomal) or permanent (chromosomal integration) according to the needs of the system.

The invention also provides antisense nucleic acid molecules which hybridise under high stringency hybridisation conditions to the nucleic acid molecules encoding the complement inhibitor molecules. In particular, the invention provides antisense nucleic acid molecules which hybridise under high stringency hybridisation conditions to nucleic acid molecules encoding the OmCI protein. High stringency hybridisation conditions are defined herein as overnight incubation at 42 °C in a solution comprising 50% formamide, 5XSSC (150mM NaCl, 15mM trisodium citrate), 50mM sodium phosphate (pH7.6), 5xDenhardts solution, 10% dextran sulphate, and 20 microgram/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at approximately 65 °C.

In a preferred embodiment, a label capable of being detected is attached to these antisense nucleic acid molecules. Preferably, the label is selected from the group consisting of radioisotopes, fluorescent compounds and enzymes.

The invention also includes transformed or transfected prokaryotic or eukaryotic host cells comprising a nucleic acid molecule, an antisense nucleic acid molecule or a vector as defined above. Where the host cells are prokaryotic cells, they preferably *E.coli* cells. Preferred eukaryotic host cells include eukaryotic yeast cells and mammalian cells.

The invention also provides a method for preparing a complement inhibitor molecule, as defined above, which comprises culturing a host cell containing a nucleic acid molecule according to the invention under conditions whereby the protein is expressed and recovering the protein thus produced. Preferably, the host cell is a yeast cell.

As discussed above in connection with functional equivalents, OmCI is believed to be a member of the lipocalin family of proteins and may exert part of its effect by binding an unidentified small ligand. Other complement inhibitor molecules of the invention and functional equivalents thereof may also exert their inhibitory effects of the pathways of complement activation by binding small ligands. The identification of these naturally-occurring ligands is desirable as they may themselves act as agonists or antagonists of the classical and/or alternative pathways of complement activation. Such naturally-occurring ligands may themselves be useful in the treatment of diseases associated with abnormally high or low complement pathway activation or may be useful starting points for the development of synthetic ligands for the treatment of such diseases. Alternatively,

naturally-occurring ligands may be useful targets for the development of additional complement inhibitor molecules that bind to them. According to a further aspect of the invention, there is provided a method of identifying a ligand of a complement inhibitor molecule or a functional equivalent thereof as described previously comprising the steps
5 of: (a) contacting the complement inhibitor molecule or functional equivalent thereof with a candidate ligand; and (b) detecting the formation of a ligand-complement inhibitor molecule complex.

Any candidate ligand may be used in this method. The candidate ligand may be isolated from, for example, cells, cell-free preparations, chemical libraries or natural product
10 mixtures. Once naturally-occurring ligands of the complement inhibitor molecules have been identified, it may be desirable to design small synthetic molecules which mimic the tertiary structure of the naturally-occurring ligands. The ability of such synthetic molecules to bind to the complement inhibitor molecules can also be tested using the method of the invention.

- 15 The complement inhibitor molecule that is used in this method may be free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. For example, the complement inhibitor molecule may be affixed to a solid support, with the candidate ligand being added subsequently. Alternatively, one or more candidate ligands may be affixed to a solid support and brought into contact with the complement inhibitor molecule.
- 20 The step of detecting the formation of a complex between the candidate ligand and the complement inhibitor molecule may be carried out by means of a label directly or indirectly associated with the candidate ligand or in an assay involving competition with a labelled competitor. In another embodiment, competitive screening assays may be used, in which neutralising antibodies that are capable of binding the complement inhibitor
25 molecule specifically compete with a candidate ligand for binding. In this manner, the antibodies can be used to detect the presence of any test compound that possesses specific binding affinity for the polypeptide.

The method of the invention may employ high throughput screening techniques known in the art to screen multiple candidate ligands simultaneously for the ability to bind to a
30 complement inhibitor molecule. For example, WO84/03564 discloses synthesising large numbers of different candidate ligands on a solid substrate, which may then be reacted

with the complement inhibitor molecule of the invention and washed. Whether or not the complement inhibitor molecule has bound to the candidate ligands may then be detected using methods that are well known in the art.

The invention also provides a ligand of a complement inhibitor molecule identified or
5 identifiable by the methods described above. Where the complement inhibitor molecule is an OmCI protein or a functional equivalent thereof, it is postulated that the ligand may be a small molecule that binds to C5 or to a C5 convertase or to a component of the MAC.

According to a further aspect of the invention there is provided a composition comprising a complement inhibitor molecule, a fusion protein comprising a complement inhibitor
10 molecule, a nucleic acid molecule comprising a nucleic acid sequence encoding a complement inhibitor molecule, or a ligand of a complement inhibitor molecule, according to the above-described aspects of the invention, in conjunction with a pharmaceutically acceptable carrier. In particular, there is provided a composition comprising an OmCI protein or a functional equivalent thereof, a fusion protein comprising an OmCI protein or
15 a functional equivalent thereof, a nucleic acid molecule comprising a nucleic acid sequence encoding an OmCI protein or a functional equivalent thereof, or a ligand of an OmCI protein or a functional equivalent thereof in conjunction with a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier", as used herein, includes genes,
20 polypeptides, antibodies, liposomes, polysaccharides, polylactic acids, polyglycolic acids and inactive virus particles or indeed any other agent provided that the excipient does not itself induce toxicity effects or cause the production of antibodies that are harmful to the individual receiving the pharmaceutical composition. Pharmaceutically acceptable carriers may additionally contain liquids such as water, saline, glycerol, ethanol or auxiliary
25 substances such as wetting or emulsifying agents, pH buffering substances and the like. Excipients may enable the pharmaceutical compositions to be formulated into tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions to aid intake by the patient. A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

30 The composition may be used as a vaccine composition and may thus optionally comprise an immunostimulating agent, for instance an adjuvant. According to a further aspect of the invention, there is provided a process for the formulation of a vaccine composition

comprising bringing a complement inhibitor molecule according to the above-described aspects of the invention, such as an OmCI protein or a functional equivalent thereof, into association with a pharmaceutically-acceptable carrier, optionally with an adjuvant. Suitable adjuvants are well-known in the art and include oil-in-water emulsion
5 formulations, saponin adjuvants, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) and other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

According to a further aspect, the present invention provides a complement inhibitor molecule, a fusion protein comprising a complement inhibitor molecule, a nucleic acid
10 molecule comprising a nucleotide sequence encoding a complement inhibitor molecule, or a ligand of a complement inhibitor molecule, as described above, for use in therapy.

The invention also provides a method of treating an animal suffering from a complement-mediated disease or disorder or preventing an animal developing a complement-mediated disease or disorder comprising administering to said animal a complement inhibitor
15 molecule, a fusion protein comprising a complement inhibitor molecule, a nucleic acid molecule comprising a nucleotide sequence encoding a complement inhibitor molecule, a ligand of a complement inhibitor molecule, or a pharmaceutical composition according to the above-described aspects of the invention in a therapeutically or prophylactically effective amount.

20 Preferably, said animal is a mammal, more preferably a human.

The term "therapeutically effective amount" refers to the amount of compound needed to treat or ameliorate a targeted disease or condition. The term "prophylactically effective amount" used herein refers to the amount of compound needed to prevent a targeted disease or condition. The exact dosage will generally be dependent on the patient's status
25 at the time of administration. Factors that may be taken into consideration when determining dosage include the severity of the disease state in the patient, the general health of the patient, the age, weight, gender, diet, time and frequency of administration, drug combinations, reaction sensitivities and the patient's tolerance or response to therapy. The precise amount can be determined by routine experimentation, but may ultimately lie
30 with the judgement of the clinician. Generally, an effective dose will be from 0.01 mg/kg (mass of drug compared to mass of patient) to 50 mg/kg, preferably 0.05 mg/kg to 10

mg/kg. Compositions may be administered individually to a patient or may be administered in combination with other agents, drugs or hormones.

The invention also provides the use of a complement inhibitor molecule, a fusion protein comprising a complement inhibitor molecule, a nucleic acid molecule comprising a
5 nucleotide sequence encoding a complement inhibitor molecule, or a ligand of a complement inhibitor molecule according to the invention in the manufacture of a medicament for treating or preventing a complement-mediated disease or disorder.

The complement inhibitor molecules of the invention, and in particular the OmCI protein and functional equivalents thereof, have potential clinical uses in the treatment of all
10 pathological conditions in which complement plays a role (Sahu and Lambris, 2000).

Preferred complement inhibitor molecules according to the invention, such as the OmCI protein and functional equivalents thereof, inhibit the classical and alternative complement pathways via inhibition of C5 cleavage by the C5 convertases of both the classical and alternative pathways. Specific inhibition of C5 cleavage by both C5 convertases blocks all
15 three pathways of complement activation leading to the generation of C5a and MAC but preserves the immune clearance and opsonisation functions of complement which depend on C3b. Such a profile may be useful for therapeutic intervention in certain diseases such as Alzheimer's Disease, rheumatoid arthritis, glomerulonephritis and delayed-type hypersensitivity disorders (Kohl, 2001).

20 For example, in a mouse model of Alzheimer's disease (AD), which is associated with prominent brain inflammation (that may be partly mediated by complement), inhibition of C3 increased the deposition rate of beta-amyloid plaques (Wyss-Coray *et al.*, 2002). Therefore inhibiting C5, but not C3, in may be beneficial in the treatment of AD.

Research into the role of C5 convertases reveals that complement inhibitor molecules of
25 the invention which inhibit cleavage of C5 by C5 convertases, such as the OmCI protein and functional equivalents thereof, will also be useful in the treatment of a wide variety of other diseases and disorders. Since no natural inhibitors of the C5 convertases have been reported, researchers have up until now targeted this step by developing inhibitory anti-C5 antibodies, inhibitory RNA aptamers and synthetic peptides that target the C5a receptor
30 (reviewed in Sahu and Lambris, 2000). Early studies, using the anti-C5 mAb BB5.1 (Frei *et al.*, 1987) have clearly established the pathological role of C5a and the MAC in various disease models including immune complex nephritis (Wang *et al.*, 1996), collagen induced

arthritis (Wang *et al.*, 1995), myocardial ischemia and reperfusion (Vakeva *et al.*, 1998) and cardiopulmonary bypass patients (Rollins *et al.*, 1998). The anti-C5 mAB (18A10) has been shown to improve neural graft survival in rats (Cicchetti *et al.*, 2002).

The complement inhibitor molecules of the invention that inhibit the classical and
5 alternative complement pathways by inhibiting the cleavage of C5 by the C5 convertases of both pathways, such as the OmCI protein and functional equivalents thereof, will therefore be of use in the treatment of these diseases and disorders in three key areas: (1) control of autoimmune diseases such as rheumatoid arthritis; (2) reduction of tissue damage due to complement following surgery; and (3) suppression of tissue rejection
10 particularly in the field of transgenic organ transplantation.

The pathology of autoimmune diseases such as rheumatoid arthritis and glomerulonephritis has many causal factors. The classical pathway of complement activation has a role in both diseases due to the presence of auto-antibodies that result in IgG and IgA antibody-antigen immune complexes within synovial fluid and glomerulus (Daha, 1993) which
15 cause inappropriate activation of complement and tissue damage. Over-expression of soluble Crry (CR1 mouse homologue) protects transgenic mice from antibody-induced acute renal failure (Schiller *et al.*, 2001). Rheumatoid arthritis immune complexes are complicated by the presence of IgM rheumatoid factors which can impede complement mediated inhibition of immune precipitation (Jarvis *et al.*, 1993) and by decreased
20 protection of synovial cells against cellular effects and lysis mediated by MAC (Kontinnen, 1996). C5 acting through the alternative complement pathway appears to have a crucial role in the K/BxN murine model of rheumatoid arthritis (Solomon *et al.*, 2002). The complement inhibitor molecules of the invention, such as the OmCI protein and functional equivalents thereof, will thus be useful in the treatment of these autoimmune
25 diseases.

Complement activation causes decrements in the myocardial function and increased coronary reperfusion pressure and lymphatic flow rate. Many of these changes may be mediated by the MAC (Homeister, 1992). Soluble CR1 protein produced by recombinant DNA technology is effective in inhibiting complement activation and consequent
30 inflammatory activities in a rat model of reperfusion injury of transient myocardial ischaemia (Weisman *et al.*, 1990). Crry reduces ischaemia reperfusion injury to mouse intestine (Rehrig *et al.*, 2001). In humans, C5 inhibition by single chain humanised

antibody h5G1.1-ScFV significantly attenuates postoperative myocardial injury, cognitive deficits and blood loss in patients undergoing cardiopulmonary bypass (Fitch *et al.*, 1999). The complement inhibitor molecules of the invention which inhibit the activity of the C5 convertases of the classical and alternative pathways will thus be useful in the prevention and treatment of postoperative myocardial injuries such as reperfusion injuries.

There is ongoing interest in classical and alternative complement inhibitors which may be effective in hyperacute allo- and xenograft organ (heart and liver) rejection (Diamond *et al.*, 1995; Thomas *et al.*, 1996; Pratt *et al.*, 1996; Tanaka *et al.*, 1996; Fiorante *et al.*, 2001; Bao *et al.*, 2002). The major immunological barrier to xenotransplantation between man and pig is a rapid rejection process mediated by preformed natural antibodies and complement i.e. by classical pathway activation. Xenogeneic organ grafts are especially susceptible to complement mediated injury because complement regulatory proteins, which normally protect cells from injury, function poorly in the regulation of heterologous complement. The complement inhibitor molecules of the invention will thus be useful in the prevention of transplant rejection. The OmCI protein and functional equivalents thereof will be particularly useful in the prevention of transplant rejection since the OmCI protein inhibits the C5 convertases of a wide range of mammalian species (rodent and primate examined to date).

The anaphylotoxin, C5a, which is produced during the conversion of C5 by C5 convertases has been found to be involved in sepsis, immune complex disease and delayed -type hypersensitivity. The OmCI protein and functional equivalents thereof, as well as other complement inhibitor proteins of the invention that inhibit C5 convertases will be useful in the treatment of these disorders. The OmCI protein and functional equivalents thereof might prove useful as an adjuvant therapy during xenotransplantation by preventing the formation of C5a and stopping deposition of the MAC that may cause upregulation of tissue factor and P-selectin expression in animal models of transplantation (Fecke *et al.*, 2002).

Other possible specific uses include: (1) prevention of platelet activation by complement during storage of platelet concentrates (Miletic and Popovic, 1993), (2) activation of complement by biomaterial surfaces during blood transfusion, (3) fertility treatment (Bedford and Witkin, 1983) and (4) protection of gene therapy retroviral vectors from lysis by natural antibody and complement during gene therapy (Rollins *et al.*, 1996).

Haematophagous arthropods, such as ticks, are extremely effective as transmitters of disease. Conventionally, techniques to control tick populations have used the treatment of animals with chemicals such as acaracides. This strategy has resulted in the development of resistant ticks, meaning that new classes of chemicals must be introduced. Furthermore, the chemicals
5 have little residual effect, meaning that they must be applied frequently. A second approach is to breed for tick-resistant animals, but the degree of resistance that results is far from ideal.

In an effort to combat parasite-transmitted diseases, a number of attempts have been made to immunise animals against ticks using extracts of whole ticks or of tick gut. Certain reports have used recombinant tick proteins (see, for example, International patent application
10 WO88/03929). However, despite such developments, the only commercially-available tick vaccines are active only against the adult stage of *B. microplus* ticks and show variation in efficacy depending on the geographical location of this species.

According to a still further aspect of the present invention, there is provided a method of vaccinating an animal against a disease or disorder transmitted by a haematophagous
15 arthropod, comprising administering to said animal a complement inhibitor molecule, a fusion protein comprising a complement inhibitor molecule, a nucleic acid molecule encoding a complement inhibitor molecule, or a composition according to the above-described aspects of the invention.

Suitable candidates for vaccination include humans and domesticated animals such as
20 cattle, goats, sheep, dogs, cats and other animals which require protection against haematophagous arthropods, especially ticks, and the infections they transmit. The vaccine may be administered singly, or in combination with other immunogens. The method of this aspect of the invention may be used to vaccinate the animal against any disease or disorder transmitted by the haematophagous arthropod. Preferably, the haematophagous arthropod
25 is a tick, preferably *O. moubata*. Diseases and disorders transmitted by ticks of the genus *Ornithodoros* include relapsing fever (Borreliosis) and West Nile virus of man, and African swine fever virus of pigs.

The invention further provides for the use of a complement inhibitor molecule according to the above-described aspects of the invention as a diagnostic tool. The identification of the
30 complement inhibitor molecules of the invention will enable researchers to study the effects of simultaneous inhibition of both the classical and alternative complement

pathways. In particular, the identification of the OmCI protein will enable researchers to study the effects of simultaneous inhibition of both the classical and alternative complement pathways via C5 convertase inhibition.

The invention also provides a method for inhibiting the classical and alternative complement pathways in a cell, tissue or non-human organism comprising administering to said cell, tissue or organism, a complement inhibitor molecule, a fusion protein comprising a complement inhibitor molecule, or a nucleic acid molecule encoding the complement inhibitor molecule, according to the above-described aspects of the invention. In particular, the invention provides a method for inhibiting the C5 convertase activity in a cell, tissue or non-human organism, comprising administering to said cell, tissue or organism, an OmCI protein or functional equivalent thereof, a fusion protein comprising an OmCI protein or functional equivalent thereof, or a nucleic acid molecule encoding an OmCI protein or functional equivalent thereof. This method will enable researchers to elucidate the role of C5 in various diseases and disorders. For example, it has been suggested that C5 may play a positive role in the prevention of asthma (Kohl, 2001). The C5 convertase inhibitors of the invention can be used to determine whether that is the case.

Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

20 Brief description of Figures:

Figure 1: Schematic diagram of classical and alternative pathways of complement activation. Enzymatic components, dark grey. Anaphylatoxins enclosed in starbursts.

Figure 2: Purification of *O. moubata* complement inhibitor (OmCI). **a.** Cation exchange chromatography. Peak containing inhibitor indicated by arrowhead. **b.** Classical haemolytic assay. Sample 1 (black bar), 100% lysis; sample 2, 0% lysis; sample 3, (cross-hatched bar) serum only; sample 4, serum plus 1µl SGE; samples 5 – 23 (grey bars) serum plus 10µl fractions 10 – 28 shown in panel **a**. Average of 3 replicates.

Figure 3: Analysis of purified OmCI by **a.** denaturing SDS-PAGE, **b.** isoelectric focusing (IEF) and **c.** high pressure liquid chromatography (HPLC). Fractions f15 and f17 in panels **a.** and **b.** are the same. Fraction f15 was recovered and analysed by HPLC, panel **c**. Size markers and isoelectric point (PI) markers are indicated at left of panels **a.** and **b.**

Figure 4: Primary sequence of OmCI. Signal sequence underlined. Cysteine residues in bold type. Nucleotide and amino acid number indicated at right.

Figure 5: Clustal X sequence alignment of OmCI with tick salivary gland proteins 2 and 3 (TSGP2 and 3) and moubatin. Identical residues are highlighted in grey (cysteines in black) and asterisked.

Figure 6: Inhibitory activity in a. supernatant and b. cell pellet of yeast clones with OmCI inserted into genome (13.1 – 13.5), and clone with vector only inserted into genome (control).

Figure 7: Expression (a) and deglycosylation (b) of yeast cell expressed rOmCI. a. SDS PAGE of fractions 9-13 from Superdex-75 gel filtration column. b. Effect of PNGaseF treatment on mobility of highly glycosylated rOmCI (fractions 9-11 in panel a). Arrows indicate PNGaseF (upper arrow) and native OmCI (lower arrow). EV504 is distantly related to OmCI and is known to be glycosylated. Size markers (kDa) indicated at left of panel.

Figure 8: Inhibition of lysis caused by classical (CH50) and alternative (AH50) pathways of complement activation by different concentrations of native OmCI. Average of 4 replicates.

Figure 9: Effect of OmCI on addition of C8 and C9 to partially formed membrane attack complex (MAC). Absorbance due to 100% and 0% lysis and in absence (PBS) and presence (SGE) of inhibitor shown. Average of 6 replicates.

Figure 10: Timecourse showing absence of effect of OmCI on classical pathway cleavage of C3a from C3 α analysed by a. denaturing SDS-PAGE and b. immunoblot with C3a specific antisera. a. Minutes (min) since start of reaction indicated. Reactions performed with (OmCI) or without (PBS) inhibitor, or in presence of 10mM EDTA. Positions of bovine serum albumin (BSA) and haemoglobin (HAE) shown. Size markers (kDa) indicated at left of panel. b. As panel a., positions of C3a and C3 α shown.

Figure 11: Effect of OmCI on classical, alternative and cobra venom factor (CVF) C5 convertase cleavage of C5a from C5 α analysed by ELISA. Picograms/ μ l C5a released measured after 100% lysis of sheep red blood cells with water, 0% lysis in GVB²⁺ only, and reactions with (OmCI) or without (PBS) inhibitor. Average of 4 replicates.

Figure 12: Effect of addition of pure C3 and C5 to C3 and C5 depleted sera on classical pathway lysis of sheep red blood cells in presence (+) and absence (-) of minimal amount of OmCI that gave complete inhibition of lysis at 1 log fold excess. Average of 4 replicates.

5 **Figure 13:** Effect of boiling on inhibitory activity of OmCI in CH50 assay.

Figure 14: Effect of pH treatment on inhibitory activity of OmCI in CH50 assay.

Figure 15: Detection of C5 binding to nOmCI. nOmCI and RaHBP2 (control) transferred to nitrocellulose were probed with I¹²⁵ labelled C3 or C5 then autoradiogramed. Protein size markers (kDa) indicated at left of panel.

10 **Figure 16:** Detection of nOmCI binding to C5 by gel filtration chromatography. Radiolabelled nOmCI **a.** with or without purified C3 and C5 (pure C3/C5) and **b.** with or without NHS, and C3 or C5 depleted sera (delta C3/C5). Protein size markers (kDa) indicated by arrows.

Examples

15 *Materials and Methods*

Materials

Sheep and rabbit red blood cells were from Tissue Culture Services., Haemolysin, pooled normal human sera (NHS) and depleted sera were all obtained from Sigma. Guinea pig sera were from in house animals. Pure C3, C4, C5, C8 and C9, and factors B and D, were
20 purchased from Calbiochem. Anti-human C3a rabbit polyclonal antisera was from Calbiochem and cobra venom factor (CVF) from Quidel. The C5a ELISA detection kit was purchased from Immuno-Biological Laboratories (IBL).

Ticks

Ornithodoros moubata ticks were reared according to Jones *et al.* (1988).

25 *Salivary gland sample preparation and purification*

Salivary glands were dissected under a microscope, rinsed briefly in cold PBS buffer (0.01M phosphate buffer and 0.15M NaCl pH 7.2) and transferred to Eppendorf vials standing in dry ice and stored frozen at -20°C. When needed, 30 pairs of salivary glands were defrosted and disrupted in 500µl PBS using a 1ml Dounce homogeniser. The
30 homogenate was centrifuged at 15K RPM in a benchtop centrifuge and the supernatant

(referred to as salivary gland extract, SGE) was collected and stored at -70°C , or tested for complement inhibitory activity and used for isolation of the active fraction.

Classical pathway of complement haemolytic assay (CH50)

Five ml of fresh sheep blood in Alsever's solution (1:1 vol/vol) were washed once in 50ml
5 Gelatin veronal barbital-EDTA (GVB-EDTA) and three times in 50ml GVB^{2+} buffer
(GVB buffer with Mg^{2+} and Ca^{2+}). The blood was diluted to a concentration of 1×10^9
cells ml^{-1} . The erythrocytes were sensitised using rabbit haemolysin, titrated as described
(Coligan, 1994). Assays were carried in a total volume of 200 μl using 100 μl 1:40 of
diluted NHS or guinea pig sera in GVB^{2+} as a source of complement and 100 μl 2×10^8
10 sensitised erythrocytes (EA) in accordance with standard protocols (Giclas, 1994). SGE,
native or recombinant OmCI (nOmCI or rOmCI) or PBS (1-5 μl) was added last, and the
reactions incubated at 37°C . At the end of the timecourse (up to 32min) whole cells were
spun down 12000 x g for 5 seconds and hemolysis measured spectrophotometrically at
412nm (Coligan, 1994). All assays were carried out at least three times.

15 *Alternative pathway of complement hemolytic assay (AH50)*

Five ml of fresh rabbit blood in Alsever's solution (1:1 vol/vol) were washed three times in
50ml GVB/Mg (10mM) EGTA buffer by centrifuging at 1500 x g for 10 mins between
washes. The rabbit blood was diluted to 2×10^8 cells ml^{-1} . NHS was diluted in GVB/Mg
EGTA buffer. The assay volume was made up to 150 μl with 50 μl prepared blood. 1-5 μl of
20 SGE, PBS, native OmCI or recombinant OmCI was added to the reactions last, and the
reactions incubated at 37°C . At the end of the timecourse (up to 60min) whole cells were
spun down 12000 x g for 5 seconds and haemolysis measured spectrophotometrically at
412nm (Coligan, 1994). All assays were carried out at least three times.

Lytic assays using sera depleted in specific complement components

25 Depleted human sera were used in accordance with the manufacturer's instructions but the
total volume of each reaction was reduced to 200 μl . Volumes and dilutions of pure
complement components that gave 90% lysis were determined empirically. Reactions were
incubated for 30mins at 37°C . All assays were carried out at least three times.

Purification of O. moubata complement inhibitor (OmCI) from SGE

30 150 μl SGE were diluted in 5ml 25mM sodium phosphate buffer pH 6.8, 50mM NaCl and
loaded onto a 1ml Q-Sepharose HP cation exchange column (Pharmacia) at a flow rate of
1ml/min. After washing with a further 10 column volumes of running buffer, bound

proteins were eluted using a 40 min 0.05 – 0.75M NaCl gradient at a flow rate of 0.5ml/min and monitored at 280 nm. One ml fractions were collected and 10µl assayed for complement inhibitory activity in 200µl total volume CH50 assays. Representative active and inactive fractions were concentrated to 50µl using Centricon 3 filtration devices
5 (Amicon), 2ml PBS was added, the fractions were concentrated to 50µl again and 1.5µl of each was run on a 4-12% Tris-Tricine denaturing SDS gel (Invitrogen). Five µl per lane of both active and inactive fractions were run on a pH 3 - 7 IEF gel (Invitrogen) and electroblotted to ImmobilonTM-P (Millipore) using 0.7% acetic acid. The membrane was stained with Ponceau-S, and major bands excised and eluted in 200µl, 50mM Tris pH 8,
10 2% Triton-X100 by vortexing for 1min and centrifuging for 10 min at 15K rpm three times. The Triton-X100 was removed by repurifying the proteins on Q-Sepahrose HP columns using the conditions described above. After Centricon 3 concentration and buffer exchange to PBS, samples were assayed for complement inhibitory activity and examined on 4-12% gels or subjected to HPLC fractionation and protein sequence analysis.

15 *Detection of C3a production during haemolytic assays*

CH50/AH50 assays were set up in a total volume of 200µl using a 1:80 final dilution of NHS or guinea pig sera with or without native OmCI. Reactions placed at 37°C were removed from the waterbath at specified time points, then spun at 12000g for 10 seconds and the supernatant removed for subsequent analysis by immunoblotting. 10µl of each
20 reduced supernatant sample was electrophoresed on 4-12% Bis-Tris gel run with MES running buffer (Invitrogen) then transferred to nitrocellulose. Confirmation of equal loading and even transfer to all lanes was judged by the intensity of the serum albumin band following Ponceau staining. C3a cleavage from C3 was detected by immunoblot using anti-human C3a rabbit monospecific antisera (Calbiochem). The nitrocellulose
25 membrane was blocked overnight with phosphate buffered saline 0.1% Tween 20, 5% non-fat dried milk (PBSTM). This buffer was used for all subsequent dilutions and washing steps unless indicated otherwise. Anti-C3a antisera was diluted 1:500 and incubated with the membrane for 2h. The membrane was then washed twice for 20 minutes before adding 1:3000 dilution of anti-rabbit alkaline phosphatase conjugate (Sigma) in PBSTM. After
30 another 2h incubation the membrane was washed twice for 5 minutes, rinsed briefly in water and 10ml BCIP/NBT purple liquid alkaline phosphatase substrate (Sigma) added.

Detection of C5a production during haemolytic assays

Haemolytic assays were performed as described for detection of C3a. A C5a ELISA kit (IBL) was used to detect cleavage of C5a from C5. To prevent cross-reaction with uncleaved C5, the C5 present in the supernatant from the haemolytic assays was
5 precipitated using the reagent provided by the kit manufacturers. The measuring range of the kit extends from 0.1 to 10µg/L. The lower limit of detection is 0.02µg/L.

Decomplementation of sera with cobra venom factor (CVF)

0.25µg CVF (0.25 µg/µl stock) and either 1µl native OmCI or 1µl PBS was added to 5µl human sera and incubated for 1 hour at 37°C. Half the CVF treated sera was added to
10 97.5µl GVB²⁺ and 100µl EA. After incubation for 20 mins at 37°C percentage lysis and concentration of C5a (see above) in reaction supernatants were determined.

HPLC of active fraction, protein sequence analysis and tryptic digestion

Twenty µl of the active fraction eluted from the IEF resolved protein was run on a Jupiter C4 column/150 x 1.0mm, and a gradient of 10-40% acetonitrile (ACN) with 0.1%
15 trifluoroacetic acid (TFA), flow rate 1 ml/min with 0.5% ACN/min increments, and monitored at 215 nm. The four close running peaks at c.53 min were transferred to Immobilon-P membrane and sequenced using an Applied Biosystems Mini-Blott cartridge. Twenty five cycles were performed on each protein.

For sequence analysis of tryptic digestion products, the major peak at 53 min (comprising
20 all four peaks observed in the first HPLC separation) was dried down in a SpeedVac and redissolved in 6M guanidine 0.5M Tris pH 8.0, then reduced and alkylated using 4-vinylpyridine. It was then re-run on the same Jupiter C4 column. No change in retention time was noted. The major peak was dried in SpeedVac and redissolved in 0.1M ammonium bicarbonate pH 8.1. Ten µl of Pierce immobilised trypsin was added and the
25 mixture incubated at 37°C for 5 hours with intermittent mixing. The mixture was then spun at 10K rpm and the supernatant was loaded on a 173a microblotting HPLC (Aquapore C18 column/100 x 0.5mm). Peaks of interest were excised from the membrane and sequenced. Fifteen cycles were performed on each protein.

Construction of O. moubata cDNA library

30 Sixty pairs of *O. moubata* salivary glands collected from nymphs after their 3rd or 4th feed were excised as described above and placed in 1ml RNeasyTM (Ambion) (in place of PBS) and stored at -20°C. mRNA was isolated using the FastTrackTM 2.0 mRNA isolation

- kit (Invitrogen) and cDNA was synthesised using a Stratagene cDNA synthesis kit (Cat # 200401-5). After fractionation into large and small cDNAs on a sepharose CL-2B column, the ethanol precipitated cDNA pellets were each resuspended in 3.5µl ddH₂O. cDNA yields were approximately 3.0ng/µl and 5ng/µl for the large and small molecules, respectively. All of the remaining large and small cDNAs were ligated into the Stratagene UniZAP XR phage vector (Cat. # 237211) and packaged with Gigapack[®] III Gold packaging extract. There were 11500 primary plaques in the large cDNA library and 480500 primary plaques in the small cDNA library. After amplification, the titres of the large and small libraries were 1.5×10^8 pfu/ml and 4×10^9 pfu/ml, respectively.
- Twenty plaques from each library were picked into 0.5ml SM buffer (0.1M NaCl, 8mM MgSO₄, 50mM TRIS.HCl pH 7.5, 0.01% gelatin) 1% chloroform and eluted from agarose plugs by vortexing. Phage insert sizes were examined by PCR using T7 (T7 5'TAA TAC GAC TCA CTA TAG 3') and T3 (5'AAT TAA CCC TCA CTA AAG 3') primers. Each 100µl reaction comprised 2µl eluted phage, 2µl 10mM dNTPs, 2µl of each primer (from stocks of 0.5µg/ml), 10µl 10X REDTaq (Sigma) PCR reaction buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 11mM MgCl₂, 0.1% gelatin), 3µl REDTaq (Sigma) DNA polymerase (1 unit/µl in 20mM Tris-HCl, pH 8.0, 100mM KCl, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Igepal CA-630, inert dye, 50% glycerol) and 79µl ddH₂O. Thermal cycling (Hybaid Touchdown thermal cycler) parameters were 1X 94°C 4min, 30X 94°C 1min, 48.5°C 45s, 72°C 90 s, and 1X 72°C 5min. Agarose gel electrophoresis of the PCR products showed that large library inserts were ≥ 1000 base pairs and small library inserts ≤ 1000 base pairs.

Cloning cDNA encoding complement inhibitor

- The N-terminal sequences determined for the two major peaks eluting at 53 min from the HPLC were used to design a degenerate primer (OF4) for use with the T7 primer (which binds to the UniZAP XR vector), to amplify the cDNA encoding the complement inhibitor. The sequence of OF4 was 5' GTAC WSN GGN WSN GAR CCN GT 3' (where: N = A or C or G or T; R = G or A; S = G or C; and W = A or T). The 100µl reaction comprised 3µl large or small cDNA library, 3µl 10mM dNTPs, 2µl T7 and 4µl OF4 (from stock of 0.5µg/ml), 10µl 10X REDTaq PCR reaction buffer, 3µl REDTaq DNA polymerase and 75µl dH₂O. Thermal cycling parameters were 1X 94°C 4min, 30X 94°C 1min, 48.5°C 45s, 72°C 90s, and 1X 72°C 5min.

Agarose gel electrophoresis revealed a range of PCR products. Two products derived from the OF4 primer were purified using a Qiaex II gel extraction kit (Qiagen) and sequenced with an ABI PRISM™ dye terminator cycle sequencing ready reaction kit and ABI sequencer (Perkin Elmer).

- 5 Conceptual translation of the largest (c.500bp) and most intense PCR product, derived from the small cDNA library using primer OF4 with T7, revealed a significant BlastX (Altschul *et al.*, 1997) match with the C-terminal sequence of the *O. moubata* platelet aggregation inhibitor moubatin (Waxman and Connolly, 1993). The sequence extended beyond the stop codon of the cDNA encoding the peptide. A reverse primer (OR1 5' GGG
10 AGG CTT TCT GTA TCC 3') matching the region beyond the stop codon was used with the T3 primer (which binds to the UniZAP XR vector) to obtain the 5' end of the cDNA. The 650bp PCR product was cloned into the pGEM®-T Easy vector (Promega) then sequenced using additional primers OR3 5' CGT CCA ATC GGT TGA AG 3' and OF6 5' GAC TCG CAA AGT CAT CAC 3'.

15 *Sequence analysis*

- Analyses were carried out using the GCG suite of programs (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, Wisc.) and also the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (<http://expasy.hcuge.ch/>). Sequences were compared with the GenBank non-redundant
20 (NR) protein database using the BlastX program (Altschul *et al.*, 1997) and searched against the Pfam (Bateman *et al.*, 2000) and SMART (Schultz *et al.*, 2000) protein domains. Multiple sequence alignment was performed with Clustal X (Jeanmougin *et al.*, 1998).

Yeast expression and purification of OmCI

- 25 The OmCI coding region was amplified by means of the polymerase chain reaction (PCR; 95 °C for 30", 50 °C for 30", 72 °C for 30"; 18 cycles), using the forward primer OM1Y (5'-ATAGAGCTCAAAATGCTGGTTTTGGTGACC-3') and the reverse primers OR7a (5'ACTGAGCGGCCCGCCTAGTGATGGTGATGGTGAT
GACCGCAGTCCTTGAGATGGGG 3' for his-tagged products) or OR6 (5' ACT
30 GAGCGGCCCGCCTAGCAGTCCTTGAGATGGGG 3' non-tagged product). The primers have built-in restriction sites, such that a *Sac* I site is added upstream of the start codon and a *Not* I site downstream of the stop codon. The product was ligated between the *Sac* I and

Not I sites of the pMETα C transfer vector (Invitrogen). The plasmid - amplified in XL1-Blue cells (Stratagene) - was transformed into the *Pichia methanolica* strains pMAD16 and pMAD11, according to the instructions of the supplier (Invitrogen). Positive clones were grown in Buffered Dextrose-complex Medium BMDY, and protein expression was induced in Buffered Methanol-complex Medium. Protein expression in the supernatant and cells of 6 positive clones was assayed every 24 hours for 5 days by CH50 lytic assay.

After 96 hours incubation, 500ml yeast cell media was centrifuged at 6370g for 15 mins and the inhibitor precipitated from the supernatant by addition of 30% (w/v) PEG-8000 and stirring on ice for 1 hour. Following centrifugation at 23700g for 1 hour the protein pellet was resuspended in 50 ml 25mM sodium phosphate buffer pH 6.8, 50mM NaCl before centrifuging at 6,000 rpm to remove insoluble material. The clarified solution was applied to a 1ml Q-Sepharose HP cation exchange column and complement inhibitory activity of fractions determined as described above. Active fractions were pooled and exchanged to 300μl PBS using Centricon 3 filtration devices (Amicon), centrifuged at 18900g for 10 minutes then applied to a Superdex™ 75 column (Pharmacia) at a flow rate of 0.5ml/min using 20mM Tris pH 7.6, 200mM NaCl as running buffer. 0.5ml fractions were monitored at 280nm and collected for 30 minutes. 5μl of each fraction was assayed for inhibitory activity and active fractions exchanged to PBS before visualisation by denaturing SDS-PAGE.

Purified rOmCI was treated with peptide N-glycosidase F (PNGaseF) in accordance with the manufacturer's instructions (New England Biolabs). Deglycosylated rOmCI was repurified by gel filtration as described above. Five inhibitory fractions were identified by CH50 and 15μl of each was run on SDS PAGE under denaturing and non-denaturing conditions.

25 *Thermostability and pH stability of native OmCI*

The minimal amount of native OmCI that inhibits classical pathway mediated cell lysis by c.90% at a 1:40 dilution of guinea pig serum was determined to be 25ng in a total reaction volume of 100μl. To examine thermostability, 1μl native OmCI (250ng) was diluted in 9μl PBS. Samples were boiled for 0, 3, 9 or 27 min, cooled rapidly on ice, and 1μl (25ng) added to 100μl CH50 assays (1:40 guinea pig serum dilution). To examine pH stability, 1μl native OmCI (250ng) was diluted in 9μl 10mM sodium acetate (pH 4.5 and 5.5), 10mM Tris.Cl (pH 7 and 8.2) or 10mM CAPS (pH 10 and 11) buffer. After incubation for

30 min at 37°C, 1µl (25ng) was added to 100µl CH50 assays (1:40 guinea pig serum dilution). Controls included 1µl of each of the buffers only in the presence and absence of 1:40 serum dilution. All assays were done in triplicate.

Method for detection of C5 binding to OmCI

5 0.5µg native OmCI and 5µg RaHBP2 were subjected to non-denaturing SDS-PAGE, then transferred to nitrocellulose and blocked overnight in PBS, 0.05% Tween 20, 5% non-fat dried milk (PBSTM). C3 and C5 were labelled with I¹²⁵ using Iodogen in accordance with the manufacturer's instructions (Pierce). Blots were incubated with 2µg I¹²⁵ labelled C3 (1440 kcpm/min), and 2µg I¹²⁵ labelled C5 (2160 kcpm/min) in 15ml PBSTM for 4 hours
10 at room temperature. After 3 x 20min washes in PBSTM at room temperature the nitrocellulose membranes were dried, and autoradiogrammed.

For gel filtration chromatography, 0.07µg I¹²⁵ labelled OmCI (1687 kcpm/min) was incubated with 2µg pure C3 or C5, or 23.8µl NHS or C3 or C5 depleted serum. PBS was added a total volume of 100µl and the mixture incubated for 10min before chromatography
15 through a Superose 12 10/30 column at a flow rate of 1ml/min PBS. 1ml fractions were collected and cpm measured at set distance from a hand held Geiger counter.

Results

Purification and identification of active fractions from O. moubata SGE

Following cation exchange chromatography, the active fraction eluted at 0.25M NaCl (Fig. 2a and b, arrow). The active fraction a control fraction (Fig. 3a) were electroblotted from an IEF gel (Fig. 3b) to a PVDF membrane which was stained with Ponceau-S. The major bands were excised, eluted, repurified by cation exchange chromatography and assayed for complement inhibitory activity. Denaturing SDS PAGE showed the inhibitory activity to be associated with a triplet of proteins with masses of around 19kDa (Fig. 3a). IEF showed
25 the inhibitory activity to be associated with a single dominant band with a pI of approximately 4.2 (Fig. 3b, upperband carryover from fraction 17). HPLC of the PVDF eluted fraction revealed four adjacent peaks (Fig. 3c). A 17 amino acid N-terminal sequence (DSESDXSGSEPVDFAFQA) obtained from the the largest peak (Fig. 3c, peak D) was used to design degenerate primers that generated a PCR product from *O. moubata*
30 cDNA library which matched the N-terminal sequence.

Primary structure of the cDNA encoding OmCI

The sequence of the full-length clone shows that OmCI is 168 amino acids long (Fig. 4). The protein has an N-terminal secretion signal comprising the first 18 residues. N-terminal sequence analysis indicates the signal peptide cleavage site is between Ala18 and Asp19.

5 The predicted molecular weight of the mature protein is 16.77kDa and the isoelectric point 4.3. There are two predicted N-glycosylation sites (Asn78 and Asn102) and twelve potential phosphorylation sites (Ser 20,22,25,84,113,115,156, Thr90, Tyr17,43,111,130, 162). However, such sites have a high probability of occurrence (protein kinase C, casein kinase II, and tyrosine kinase sites) and a site prediction may not necessarily indicate a

10 genuine modification.

The primary sequence of OmCI shows 58% identity to tick Salivary Gland Proteins 2 and 3 (TSGP2 and 3) of the soft tick *Ornithodoros savignyi* (Mans *et al.*, 2001), and 49% identity to moubatin from *Ornithodoros moubata* (Waxman and Connolly, 1993). All the cysteine residues, and therefore presumably the disulphide bridging pattern, are conserved

15 in these four proteins (Fig. 5). The alignment shows that OmCI has two obvious short amino acid insertions: SESD at the amino terminus and PD about two-thirds of the way through the sequence of the mature peptide (Fig. 5). The primary sequence does not have a significant match with any other any other sequences in public databases including the anti-complement protein of *I. scapularis* (Valenzuela *et al.*, 2000). Moubatin, and TSGP2

20 and 3 are believed to be members of the lipocalin family of beta barrel forming proteins that include the histamine binding protein family of tick specific proteins (Paesen *et al.*, 2000).

Expression and purification of recombinant (r) OmCI

The 6 positive yeast clones assayed exhibited variable levels of OmCI expression (Fig. 6a

25 and b). In all cases where expression was detected, inhibitory activity continually increased through to the final assay point on day 5. Approximately 90% of the expressed protein was in the supernatant (Fig. 6b). Clone 13.1 appeared to give the highest expression levels and was used for subsequent expression studies. Following PEG precipitation and two chromatography steps partially purified active rOmCI is present in heavily glycosylated

30 (Fig. 7a, fractions 9, 10 and 11) and unglycosylated forms (Fig 7a, fractions 12 and 13). The glycosylated form was shown to correspond to the unglycosylated form by treatment with PNGaseF (Fig. 7b). Glycosylated and unglycosylated or deglycosylated rOmCI and

native OmCI are equally active in CH50 assays (data not shown). The final yield of rOmCI was approximately 0.3µg/ml of media.

Mechanism of action of OmCI

OmCI inhibits both complement pathways. However while the classical pathway can be
5 entirely inhibited, even excess OmCI inhibits lysis of red blood cells by the alternative pathway by no more than 80% (Fig. 8).

OmCI does not prevent incorporation of C8 and C9 into preformed C5b-7 or C5b-8, respectively (Fig. 9). Nor does it affect the rate of C3α cleavage to yield C3a by either the classical or the alternative pathways (Fig. 10). OmCI does prevent production of C5a from
10 C5 by both pathways (Fig. 11). Excess pure C5, but not C3 out-competes, the OmCI inhibitor in the classical haemolytic assay (Fig. 12). OmCI does not prevent decompensation of sera by CVF (data not shown). Nor does it prevent C5a production by the CVF C3/C5 convertase (CVFBb) (Fig. 11).

15 *Thermostability and pH stability of native OmCI*

Boiling OmCI for up to 9 minutes did not have a significant affect on the inhibitory activity of the protein, although by 27 minutes inhibitory activity had decreased (Fig. 13). Native OmCI was unaffected by exposure to alkaline buffers up to pH 11 (Fig. 14). Exposure to buffer of pH 4.5 markedly decreased the inhibitory activity of OmCI (Fig 14).
20 Silver stained gels showed that this was not simply due to precipitation of OmCI at this pH (data not shown).

Detection of C5 binding to OmCI

Western blotting with I¹²⁵ labelled C3 and C5 indicates that OmCI binds directly to C5 but not to the related protein C3 (Fig. 15).

25 Additional evidence for a direct interaction between OmCI and C5 was obtained by gel filtration chromatography. An apparent mass shift in a proportion of the I¹²⁵ labelled nOmCI was observed in the presence of purified C5 but not C3 (Fig. 16a). A similar mass shift was evident in the presence of NHS and C3 depleted sera but not C5 depleted sera (Fig. 16b). The mass shift was maintained in the presence of 1M NaCl but not 2M NaCl
30 indicating a strong electrostatic interaction between the inhibitor and C5 (data not shown).

Discussion

Relationship to other proteins and to known complement inhibitors

OmCI is most closely related to tick salivary gland proteins 2 and 3 (TSGP2 and 3) of the soft tick *O. savignyi* (Mans *et al.*, 2001) and the platelet aggregation inhibitor moubatin
5 (Waxman and Connolly, 1993). It has not been shown, or suggested, that any of these three proteins (Fig. 5) inhibit complement. The two small amino acid insertions present in OmCI but not in the closely related proteins (Fig. 5) are obvious sites to focus on in future mutagenesis studies to define complement binding sites in OmCI.

TSGP2 and 3 have 95% amino acid identity and have been proposed to have roles in the
10 granule biogenesis of tick salivary glands (Mans *et al.*, 2001). TSGP2 is toxic to mice; TSGP3 is not (Mans *et al.*, 2002). OmCI is highly unlikely to be a toxin since *O. moubata* is non-toxic (Astigarraga *et al.*, 1997) whereas *O. savignyi* causes sand tampan toxicoses in a wide range of mammals (Mans *et al.*, 2002). Furthermore, inoculation of guinea pigs with 100µg of purified native OmCI, in the process of raising antisera, caused no obvious
15 pathophysiological effects (personal observation).

OmCI is probably a member of the lipocalin family of proteins that include the histamine binding protein family of tick specific proteins (Paesen *et al.*, 2000). Lipocalins predominantly bind small, hydrophobic, extracellular ligands within their beta-barrel structures. However, the histamine binding protein of the tick *Rhipicephalus*
20 *appendiculatus* has significant structural differences from normal lipocalins that enable it to bind hydrophilic molecules (Paesen *et al.*, 1999; Paesen *et al.*, 2000). It is not yet known whether OmCI binds any small ligands.

The primary sequence of OmCI has no detectable similarity to complement control protein (CCP) domains (multiple *c.*60 amino acid repeats) which form many of the bodies own
25 complement inhibitors including factor H, C4BP, CR1, CR2, MCP and DAF). Nor is it similar to any other known complement inhibitors in public databases including Isac, the salivary complement alternative pathway inhibitor protein of *Ixodes scapularis* (Valenzuela *et al.*, 2000). It is also unrelated to the N-terminal sequence of *O. moubata* antigen 20A1 (Baranda *et al.*, 2000) which was proposed to be the factor responsible for
30 the potent complement inhibition previously observed in the SGE's of *O. moubata* and *O. erraticus* (Astigarraga *et al.*, 1997).

Mechanism of complement inhibition

- Both glycosylated and deglycosylated rOmCI expressed in yeast are as potent as the native protein purified from SGE. C-terminal histidine tagged OmCI expressed in insect cells is not as potent (data not shown). OmCI inhibits both the classical and alternative pathways of complement activation of both humans and guinea pigs and presumably other mammals as well. This property should prove useful in defining precisely how OmCI works, and will be invaluable in the development of animal models of complement mediated diseases where the species specificity of present C5 inhibitors have hampered *in vivo* studies using rodents (Link *et al.*, 1999).
- OmCI does not inhibit either the classical (C4bC2a) or the alternative (C3bBb) C3 convertase since it has no effect on the rate of C3 α cleavage (Fig. 10). OmCI does prevent production of C5a from C5 (Fig. 11). Since excess C5 out-competes the OmCI inhibitor (Fig. 12) functional classical (C4bC2aC3b) and alternative (C3b²Bb) C5 convertases must be formed in the presence of the tick inhibitor. OmCI is unlikely to be a direct serine protease inhibitor of the convertase catalytic components C2a and Bb or it would prevent C3a as well as C5a production. The inhibitor does not prevent C5a production by the CVF C3/C5 convertase (CVFBb) (Fig. 11) which suggests OmCI does not bind C5 and block the C5a cleavage site. The latter finding does not exclude the possibility that OmCI binds to a site on C5 that prevents binding to the normal serum C5 convertases but not to the CVF convertase (Sandoval *et al.*, 2000).

Two independent lines of evidence suggest OmCI activity is mediated through direct binding to C5 (Fig. 15 and Fig. 16).

- Although OmCI inhibits both complement pathways, even with excess inhibitor the alternative pathway is inhibited by at most 80% (Fig. 8). This is explicable in terms of the different C5 convertases used by the classical (C4bC2aC3b) and alternative (C3b²Bb) pathways, but the mechanism remains to be explored.

In summary, OmCI probably either binds C5 and prevents it interacting with the C5 convertases or binds the C5 convertases and C5 and prevents C5 cleavage. Presently we have no compelling evidence supporting one possibility over the other.

Thermostability and pH stability of native OmCI

OmCI is thermostable but activity begins to be lost after being boiled for 27 minutes. OmCI appears to be sensitive to acid and insensitive to alkali. Both prolonged boiling and exposure to acid probably induce conformational changes that inactivate the protein.

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